EXHIBIT 8b

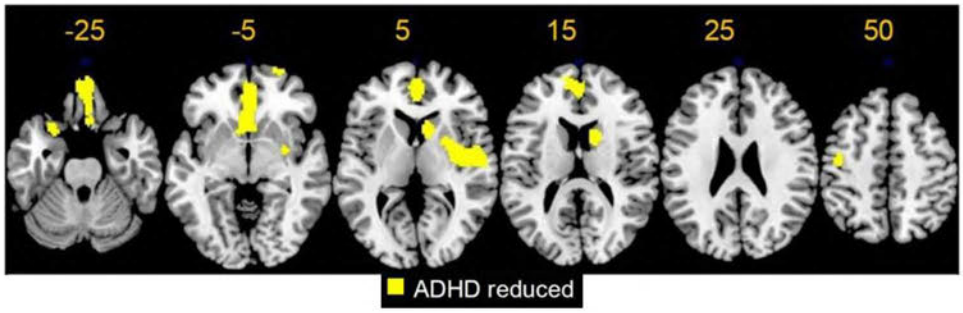

Figure 13. Structural comparison of ADHD vs. typically developing brain. Reproduced from Lutiko et al., 2020.

Some volumetric changes observed in childhood normalize later in life, but the changes in many measures remain fixed. For example, a longitudinal study of ADHD patients showed that reduced dorsal surface area and lower basal ganglion volumes shown in adolescence did not normalize with aging (Faraone et al., 2015). Additionally, ADHD subjects experienced a progressive contraction of ventral striatal surface area with aging, whereas control subjects experienced increased surface area with age (Faraone et al., 2015).

"Remission of ADHD has been associated with normalization of abnormalities as measured by activation during functioning imaging tasks, cortical thinning, and structural and functional brain connectivity. Although these data could be taken to suggest that the age-dependent decline in the prevalence of ADHD might be due to the late development of ADHD-associated brain structures and functions, most patients with ADHD do not show complete developmental 'catch up'. Indeed, widespread deviations in cortical thickness persist in many adults with ADHD" (Faraone et al., 2015).

With respect to functional differences in ADHD compared to typically developing controls, meta-analyses demonstrate underactivation in lateral and frontostriatal networks (i.e., right anterior cingulate cortex/anterior insula/inferior frontal gyrus/supplementary motor area, and striatum) (Hart et al., 2013; Norman et al., 2016; Lukito et al., 2020).

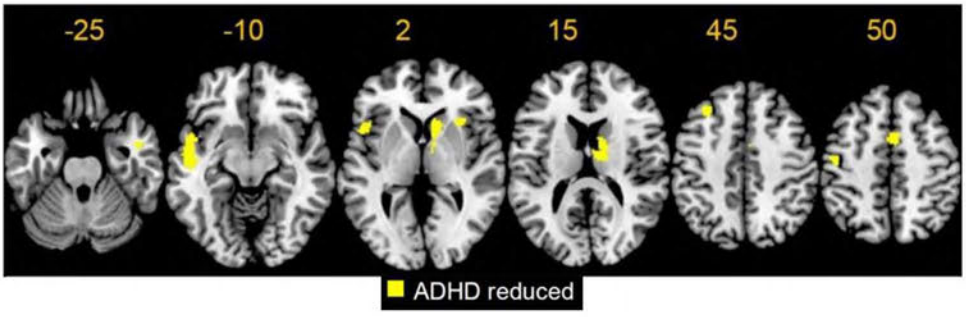

Figure 14.fMRI comparison of ADHD vs. typically developing brains during cognitive control. Reproduced from Lutiko et al., 2020.

The meta-analysis by Lukito revealed ADHD underactivation in the right inferior frontal and striatal regions during motor response inhibition. This finding was consistent with previous findings of underactivation in the right inferior frontal gyrus and caudate, which are key to

cognitive control processes including attention, decision-making, and inhibitory control (Hart et al., 2013; Rae et al., 2014; Norman et al., 2016; Zhang et al., 2017; Lukito et al., 2020).

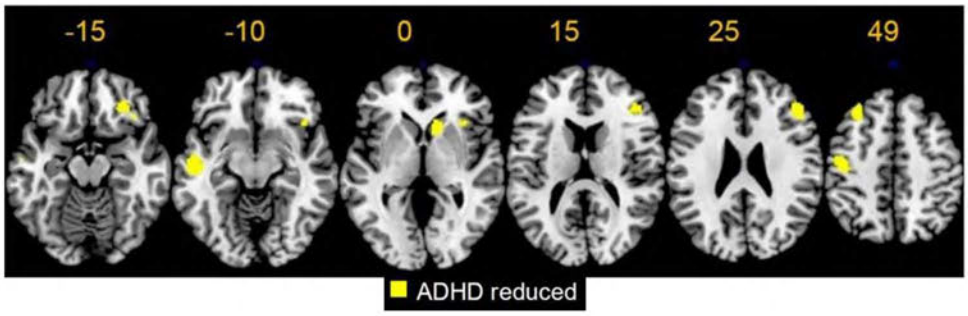

Figure 15. fMRI comparison of ADHD vs. typically developing brains during motor response inhibition. Reproduced from Lutiko et al., 2020.

Baker et al., 2020 studied the association between prenatal APAP exposure, determined by presence and level of APAP in meconium, and ADHD in children. The potential for mediation of the association between prenatal APAP exposure and hyperactivity was tested by reference to resting-state brain activity from fMRI. Compared to children with no APAP detected in meconium (n = 23), children who had detectible levels of meconium APAP (n = 25) showed increased negative connectivity between the medial prefrontal cortex gyrus and regions of bilateral precentral and postcentral gyri, superior parietal lobules, and supramarginal gyri, as well as increased negative connectivity between the left lateral prefrontal cortex and portions of the right precentral and frontal gyrus (Baker et al., 2020). These findings involve many of same regions identified in meta-analyses of structural and functional MRI of ADHD.

2. Altered Neurotransmission in ADHD

Genome-wide association studies have found several genetic variants involving neurotransmission and neurodevelopment are implicated in ADHD. This data, which provides important information about ADHD etiology and pathophysiology, identifies six primary neurotransmitters/neuroreceptors that play an important role in the pathophysiology and pathogenesis of ADHD: dopamine, serotonin, norepinephrine, acetylcholine, and glutamate/GABA.

Dopamine. The dopaminergic pathway plays a vital role in the development and manifestation of ADHD. Dopamine signals regulate emotion, reward, locomotion, complex behavior, and cognition, and dysfunctional dopamine signaling in the forebrain leads to inappropriate or deficient attention (Kessi et al., 2022). Numerous studies have shown that persons with ADHD have decreased dopamine receptor density in several brain regions, and studies of children with ADHD have identified the frontal cortical regions, which are rich in dopamine, as sites related to ADHD (Kessi et al., 2022).

Serotonin. Patients with ADHD have shown relatively low platelet serotonin levels. Serotonin signaling regulates cognition, behavior, and immunity, and disrupted serotonergic development can alter brain function resulting in anxiety, depression, impulsivity, irregular appetite, and violence (Corominas-Roso et al., 2013). Biochemical and pharmacological evidence

demonstrate that reduced serotonergic function is linked to impulsivity, which is a prominent feature in ADHD (Kessi et al., 2022).

Norepinephrine. Adults diagnosed with ADHD have shown reduced norepinephrine availability in brain regions relevant to attention, which is more pronounced in the right hemisphere (Ulke et al., 2019). Norepinephrine signaling regulates cognition, memory, and stress regulation.

GABA and Glutamate. As discussed above, Glutamate is involved in several neurological functions, including synaptic plasticity, regulating dopamine signaling, memory formation, learning, and brain development, (Kessi et al., 2022). Children with ADHD have shown higher levels of glutamate and lower levels of GABA compared to controls, and this imbalance leads to inattention, hyperactivity, and impulsivity (Courvoisie et al., 2004).

Acetylcholine. Cholinergic dysfunction is associated with many neurological conditions, including ADHD (English et al., 2009). Acetylcholine is critical to the regulation of several CNS functions, including attention, learning, memory and motivation – all of which are dysregulated in persons with ADHD (Johansson et al., 2013).

C. ASD AND ADHD SHARED CHARACTERISTICS

ASD and ADHD share some symptomology and are often diagnosed together. A significant proportion of children with ASD also meet the criteria for ADHD. Both ADHD and ASD are comprised of symptoms characterized by difficulties with attention and social interaction. Children diagnosed with ADHD may have difficulty focusing on tasks, may be easily distracted, and may struggle with impulse control. Similarly, children with ASD may have difficulty with attention and may exhibit repetitive behaviors or restricted interests. Additionally, both disorders can lead to problems with social interaction and communication. Children with ADHD may struggle to notice social cues or may interrupt others during conversations. Children with ASD may have difficulty understanding social norms or may struggle to make eye contact during conversations.

One hallmark of both ASD and ADHD is sex-specific differences in the way each disorder typically manifests. These sex-specific features are present in both humans and their corresponding animal models (Berger & Sagvolden, 1998; Jeon et al., 2018; Mahendiran et al., 2019). The complete mechanisms of such sex differences are not fully characterized but can reflect hormonal, genetic, societal, clinical, and even immunological differences.

D. SHARED ETIOLOGY OF NEURODEVELOPMENTAL DISORDERS

As discussed above, ASD and ADHD are often co-diagnosed, and research has identified shared genetic and environmental factors that contribute to the development of both ASD and ADHD. ASD and ADHD are both individual phenotypes that may have a wide range of overlapping etiologies, including genetic and environmental factors.

Several genes have been identified that are associated with an increased risk for both ADHD and ASD, including genes involved in neurotransmitter signaling and neuronal development (Satterstrom et al., 2019; Mattheisen et al., 2022). However, as discussed in more

detail later in this report, the presence of a gene, or group of genes, which create a propensity for ASD and/or ADHD is not the sole determiner of the ultimate phenotype, meaning that many people carry variants in these genes and are ultimately not diagnosed with either disorder.

Environmental factors, such as prenatal exposure to toxins and toxicants, have also been linked to an increased risk for both disorders. Brain imaging studies and neurochemical analysis have also identified significant shared abnormalities in individuals with ADHD and ASD, particularly in regions of the brain involved in social cognition, attention, and executive functioning (see, e.g., Rommelse et al., 2010). Analyses of the effect of APAP on neurodevelopment have also specifically pointed out that shared environmental risk factors for ADHD and ASD may have a transdiagnostic feature (Kim et al., 2020). These findings suggest that there may be common underlying neural mechanisms that contribute to the development of both disorders.

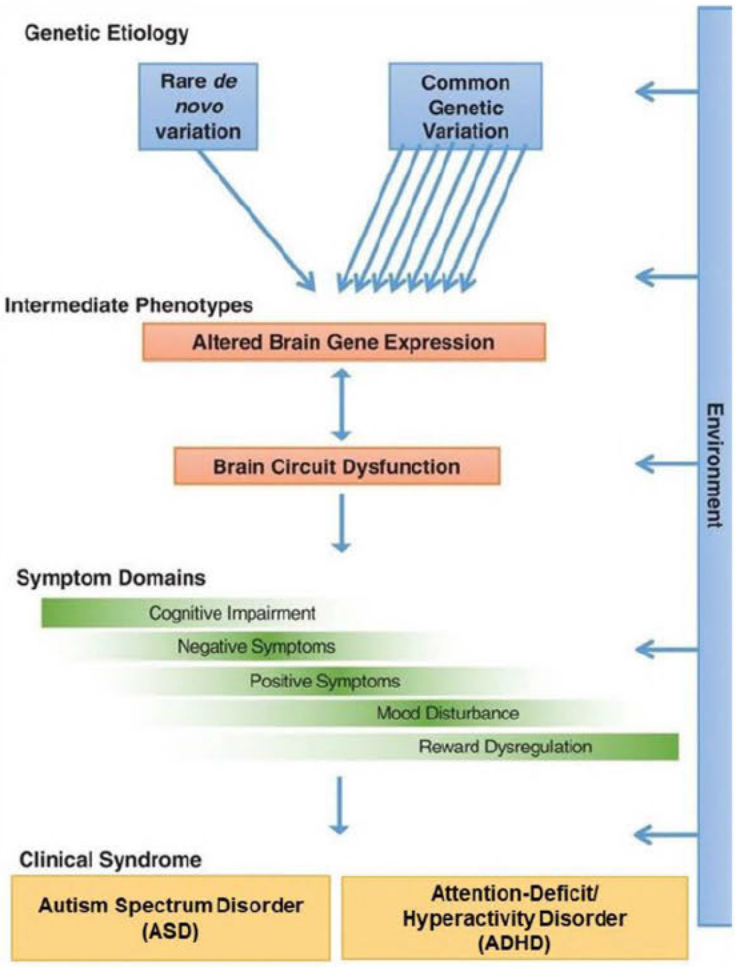

Figure 16. Depiction of how genetic and environmental etiological factors (blue boxes) converge on molecular, cellular and synaptic "intermediate phenotypes" that underlie symptom domains and clinical phenotypes that overlap, cellular and synaptic "intermediate phenotypes" that underlie symptom domains and clinical phenotypes that overlap and share upstream contributors.
Modified from Gandal et al., 2018.

E. Genetic Susceptibility to Neurodevelopmental Disorders

1. Genes and Gene Expression

As discussed above, genes play an important role in determining whether an individual person may develop a neurodevelopmental disorder. However, gene expression, a delicate and easily disrupted process, is perhaps more central. Genes and gene expression are two distinct but closely related concepts.

Genes themselves are the segments of DNA that carry hereditary information for protein sequences. Variation in gene sequence affects protein sequences capable of contributing to pathological disease states, or vulnerabilities to disease states. Gene variants can be inherited or they can emerge in parental germ cells (de novo mutations) or even arise post conception in what are termed somatic mosaic mutations.

Gene expression encompasses the complex processes by which the information within a gene is used to generate functional products, often a protein or RNA molecule. While an individual organism has many genes, the only observable traits or characteristics that are ultimately expressed are phenotypes.

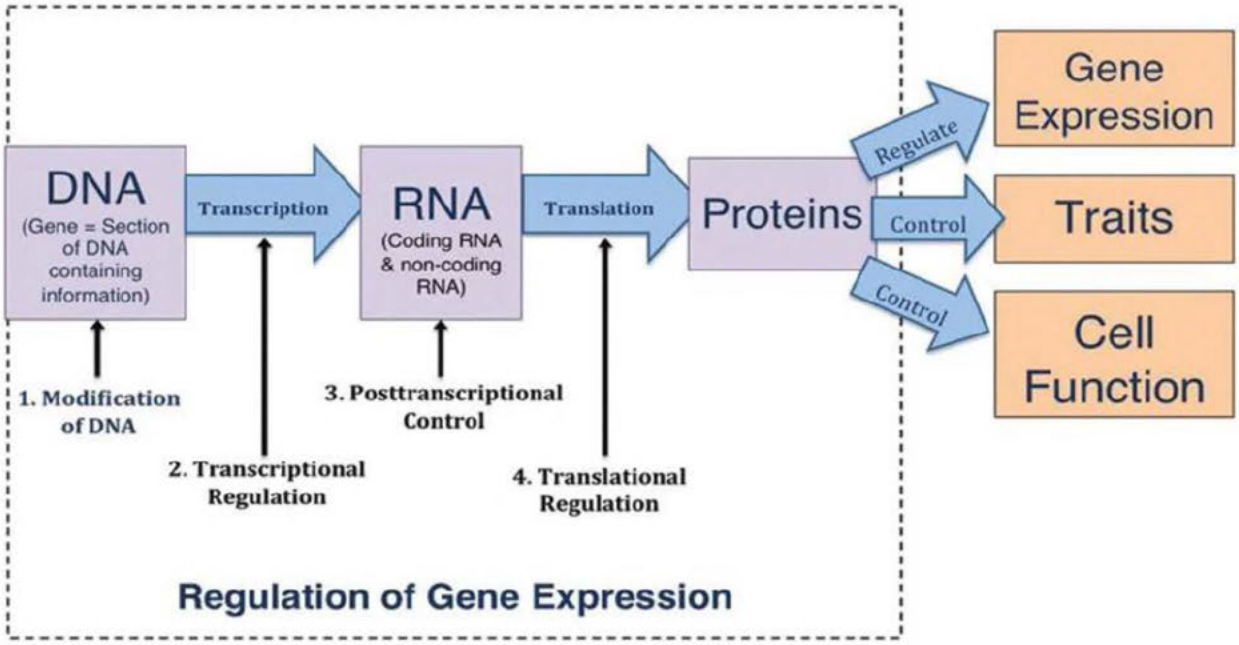

Figure 17. Graphical depiction of how the information molecules of a cell ensure that a cell does what it is supposed to do under a normal, basal state, but also can respond to stimuli as needed, in an adaptive manner. Reproduced from https://microbenotes.com/gene-expression/.

Phenotypes are the outward manifestations of the underlying genetic and environmental influences on an organism. While an individual organism may have any number of underlying genes, the ultimate outcome for that organism (the phenotype) is determined through a combination of gene

expression and other environmental factors. Gene expression is tightly regulated to ensure that specific genes are activated or repressed in a precise and coordinated manner. This regulation allows cells to respond to diverse signals and adapt to environmental changes. Gene expression control occurs at multiple levels, including transcriptional regulation through interactions between transcription factors and gene promoters or enhancers. types. The timing and way genes are expressed allow the diverse array of cell types and tissues to form in order generate an organism with its component parts. Flexibility in gene expression programs is necessary to respond to environmental and homeostatic changes, but disturbances to adaptative and normal developmental transcriptional programs can underlie pathologies independent of, or in ways that interact with gene sequence.

Gene expression involves sequential steps. Gene expression begins with transcription, wherein a complementary RNA molecule, known as messenger RNA (mRNA), is synthesized from a DNA template. Within our cells, mRNA's role is to carry genetic information from DNA to the ribosomes, where it serves as a template for protein synthesis. Once the mRNA molecules carry the genetic code from the nucleus to the cytoplasm, translation begins. During translation, ribosomes and transfer RNA (tRNA) molecules facilitate the synthesis of a protein by interpreting the mRNA template. Once the process of transcription is complete, regulation continues through mechanisms such as microRNAs, which modulate mRNA translation. Post-translational modifications, such as phosphorylation or acetylation of proteins, can also influence gene expression.

Gene expression refers to the process of converting genetic information into functional molecules. By contrast, genetic susceptibility refers to the increased likelihood of developing a trait or disease (i.e., a phenotype) based on specific genetic variations. Genetic susceptibility can be influenced by genetic variations, including mutations or polymorphisms in specific genes or regions of the genome. These variations can impact an individual's susceptibility to various disorders, including neurodevelopmental disorders, and individual toxic exposures.

As the diagram adapted from Gandal et. al. (Fig 16) illustrates, altered gene expression disrupts normal brain circuitry. That disruption may manifest in phenotypes or behaviors that fall into any number of categories in terms of a formal (human) diagnosis. As discussed above, although the diagnostic criteria in humans have a place, they are fundamentally a clinical construct with limited utility in terms of determining the etiology of a specific disorder, particularly for highly heterogeneous disorders like ASD and ADHD.

With regard to APAP and gene expression during neurodevelopment in particular, there is ample evidence that APAP disrupts the delicate process of gene expression in the developing brain (see, e.g., Baker et al., 2023).

2. Specific Syndromes

Neurodevelopmental syndromes are a distinct subset of neurodevelopmental disorders characterized by specific clinical features and associated genetic abnormalities. These syndromes, such as Down syndrome, Fragile X syndrome, Rett syndrome, and Angelman syndrome, among others, are characterized by well-defined, highly penetrant genetic etiologies and often exhibit unique clinical presentations. Unlike ASD or ADHD, neurodevelopmental syndromes typically

demonstrate less heterogeneity and are distinguished by their genetic causes and clinical profiles. Syndromes are generally distinct in that the etiology or cause of the disease or disorder is well defined.

3. Neurotoxins and Neurotoxicants

Neurotoxins and neurotoxicants are substances that can harm or impair the development and function of the nervous system. The terms neurotoxin and neurotoxicants are often used interchangeably but have slightly different meanings depending on the context Neurotoxicologists commonly differentiate between substances that have biological origins as neurotoxins (such as BOTOX or Botulinum toxin which is a chemical derived from bacteria) versus toxicants or neurotoxicants, used more broadly (and which will be used hereafter) to also include those that are synthetic or result from anthropogenic activities, such as pesticides, industrial chemicals, or drugs. Neurotoxicants can damage the nervous system at any age, but exposure during critical periods of neurodevelopment can disrupt normal brain development, leading to an increased risk of neurodevelopmental disorders. Substances that are particularly harmful during periods of neurodevelopment are sometimes referred to as developmental neurotoxins or developmental neurotoxicants.

The neurodevelopmental disorders caused by toxic substances encompass a wide range of conditions characterized by atypical brain development, altered neuronal connectivity, and subsequent cognitive, behavioral, and functional impairments.

Various environmental factors contribute to neurotoxicant exposure, including industrial pollutants, heavy metals (e.g., lead, mercury), pesticides, certain medications, and maternal substance abuse. The developing brain is particularly vulnerable to the adverse effects of neurotoxicants as it undergoes intricate processes of neuronal proliferation, migration, cell fate determination, synaptogenesis, and myelination.

Neurotoxicants can exert harmful effects through multiple mechanisms. They may interfere with neurotransmitter systems, disrupt synaptic connections, alter cellular signaling pathways, induce oxidative stress and inflammation, and interfere with the balance of essential neurodevelopmental processes. These disruptions can result in long-lasting or permanent changes in brain structure and function, contributing to the development of neurodevelopmental disorders.

VII. PRECLINICAL RESEARCH

Preclinical studies are laboratory studies that include animal models (in vivo), cell culture (in vitro), computational (in silico), and ex vivo (organs or tissues studies outside of the body). In general, preclinical studies are performed prior to human clinical trials to understand the efficacy and/or toxicity of a device or compound. However, preclinical research is also performed when the safety of an approved or existing compound is unclear or called into question. Preclinical research is essential to prevent waste of financial and clinical trial resources and is performed in lieu of invasive or unethical human experimental approaches to avoid side effects or other serious adverse effects of test agents, drugs, or devices and for approved substances whether they are agrochemicals, industrial agents, approved therapeutics, or legacy drugs or chemicals. Subtypes of Preclinical Research include:

In Vivo. In vivo means experiments done in or using the whole body of a whole organism. A drug administered "in vivo" means it is given systemically to the intact animal. A main benefit of in vivo preclinical research is that it models the human condition most directly and can evaluate the systemic effects across organ systems as well as health and behavioral influences. In biomedical pursuits, we are attempting to understand how chemicals make us sick or healthy. An intact organism with its varied and interconnected systems (circulatory, endocrine, nervous, gastrointestinal, etc.) will always be the most ideal way of understanding the full impacts of a drug. However, ethics considerations are such that we always want to reduce, refine, and replace in vivo animal model approaches where we can. Animal models are discussed in more detail later in this report.

In Vitro. In vitro means in culture, or cultured cells or tissue that are grown in plates or dishes in an incubator, outside of the body. Often immortalized cell lines (i.e., cells that have been manipulated to proliferate indefinitely) are used as they are robust and well characterized. Primary cells are cells that are harvested directly from tissues and grown. The benefits of in vitro experiments are that researchers can use relevant cell types but avoid issues surrounding drug delivery. They also reduce the numbers of animals used in preclinical research and create opportunities for higher dimensionality for doses, duration, chemicals, and readouts. There are instances where in vitro approaches will not be sufficient.

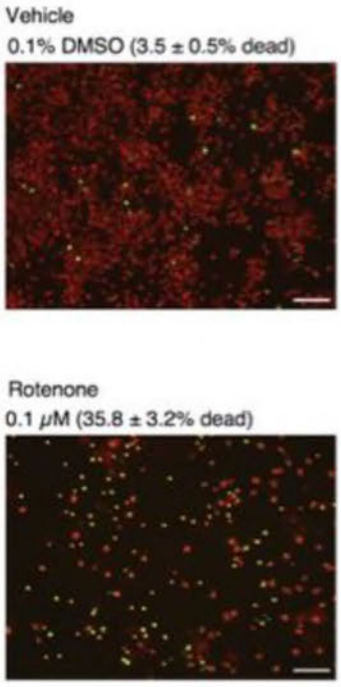

Figure 18.

Cell viability or the inverse, cell death (as in the Figure on the left which shows primary cortical neurons grown from embryonic mouse brains [data from Pearson et al., 2016 *Nature Communications*]) can be evaluated in vitro. The growth of the cells and function of the cells can be measured. Other metrics such as enzyme activities, or in the case of nervous system cells, electrochemical communication or synapse formation are readily evaluated in cultured cells. The advent of human induced pluripotent stem cell (iPSC) technologies has created the opportunity to grow human induced stem cell-like cells from human participants which can be tested for toxicological effects as stem cells or in terminally differentiated cell types such as neurons. In vitro technologies are rapidly advancing, but they do not replicate the entirety of an intact organism with other organ systems (circulatory system, biliary system, renal system, immune system, etc). Toxicants and drugs do not act in a vacuum of one cell type or one system so in vitro testing must consider its inherent limitations.

In Silico. In silico means computational. These are analyses using a computer that are used to predict properties of chemicals where data from physical experiments is lacking or non-existent. Read across tools can be used to fill data gaps about chemical toxicity for assay outcomes where they have not actually been tested. Using quantitative structure activity relationships (QSAR), physical-chemical properties, and other variables can be used to in regressions and machine learning algorithms to classify and predict toxicological endpoints for thousands of environmental chemicals and drugs. Bioinformatics tools, data visualizations, data reduction techniques, data integration and other

statistical methods are also computational or in silico approaches that can aid in determining the toxicity of a chemical.

VIII. BIOLOGICAL PLAUSIBILITY

The preclinical studies reviewed in this report relate to the biological plausibility that APAP causes neurodevelopmental disorders. Biological plausibility concerns whether, based on existing knowledge about human biology and disease pathology, an exposure could plausibly cause a disease. Biological plausibility is one of the factors enumerated by Sir Austin Bradford Hill to aid epidemiologists, who study the incidence of disease in human populations, in determining causation.[5] In this case, numerous observational human studies (i.e., epidemiological studies) have found a consistent association between APAP exposure in utero and neurodevelopmental disorders, most frequently ASD and ADHD (See expert report of Andrea Baccarelli, MD, PhD, MPH). Those studies include multiple careful study design features designed by researchers to make the results as clinically relevant as possible and to reduce confounding, but they do contain limitations as described by their authors (and as typically required by the peer review process) (See expert report of Andrea Baccarelli, MD, PhD, MPH).

Though the most conclusive answer to the causation inquiry would be obtained through a randomized controlled trial (RCT), a RCT would not be feasible in this situation for several reasons. Most importantly, it would be unethical to subject pregnant women to treatment with a drug suspected to cause neurodevelopmental disorders in offspring. Causation can be established in the absence of randomized control trials (see, e.g., the causal link between smoking and lung cancer and newer causal epidemiological methods [e.g., Mendelian randomization, reviewed in Sanderson et al., 2022]). Biologic plausibility based on preclinical studies plays an especially

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The Bradford-Hill considerations provide a framework within epidemiology to assess causation and include:

1. **Strength** (effect size): A small association does not mean that there is not a causal effect, though the larger the association, the more likely that it is causal.
2. **Consistency** (reproducibility): Consistent findings observed by different persons in different places with different samples strengthens the likelihood of an effect.
3. **Specificity**: Causation is likely if there is a very specific population at a specific site and disease with no other likely explanation. The more specific an association between a factor and an effect is, the bigger the probability of a causal relationship.
4. **Temporality**: The effect has to occur after the cause (and if there is an expected delay between the cause and expected effect, then the effect must occur after that delay).
5. **Biological gradient**: Greater exposure should generally lead to greater incidence of the effect. However, in some cases, the mere presence of the factor can trigger the effect. In other cases, an inverse proportion is observed: greater exposure leads to lower incidence.
6. **Plausibility**: A plausible mechanism between cause and effect is helpful (but Hill noted that knowledge of the mechanism is limited by current knowledge).
7. **Coherence**: Coherence between epidemiological and laboratory findings increases the likelihood of an effect. However, Hill noted that "... lack of such [laboratory] evidence cannot nullify the epidemiological effect on associations".
8. **Experiment**: "Occasionally it is possible to appeal to experimental evidence".
9. **Analogy**: The effect of similar factors may be considered.

critical role in situations where such testing is impossible or unethical, and an essential role even when RCT is possible.

Because observational human studies do have limitations, animal models and other preclinical studies are critical and commonly used to determine causation. While epidemiological studies may have hidden confounders, researchers performing preclinical tests in animals exert substantial control over all variables and thereby mitigate confounding and bias concerns. They can also help show the plausible mechanisms of action for a particular injury.

As described below, the preclinical research relevant here demonstrates the biological plausibility that prenatal APAP exposure causes neurodevelopmental disorders, including ASD and ADHD, in offspring.

IX. ANIMAL MODELS IN PHARMACEUTICAL AND NEURODEVELOPMENTAL RESEARCH

In vivo studies—in other words, animal studies—are a primary source for preclinical data of biological possibility, including for studying ASD and ADHD. Although these are human disorders, other animals, such as rats and mice, express analogous symptoms as humans with those disorders. Thus, the disorders can be "modeled" in these animals, enabling researchers to study potential causes of these symptoms.

The phrase "animal model" refers to the use of non-human animals to replicate key features of human diseases, conditions, or related physiological processes. These models are created by genetic manipulation, selective breeding for naturally occurring characteristics that resemble human disease, or exposure to environmental factors or inducing lesions that mimic human disease (Wickens et al., 2011). The validity of a proposed animal model is evaluated primarily by reference to three criteria: (1) face validity (i.e., the degree to which the model mimics the fundamental behavioral characteristics of the human disorder); (2) construct validity (i.e., the degree to which the animal expresses the known etiology and pathophysiology of the human disorder); and (3) predictive validity (i.e., the degree to which the animal's response to treatment corresponds to human treatment response) (Belzung et al., 2011). Rodent models of ASD, for instance, can demonstrate good face validity, moderate to good construct validity, but cannot demonstrate good predictive validity because there are no curative treatments that resolve the symptoms of ASD that can be taken to the animal model to validate it. This does not invalidate their use, but it does provide an example of how substantial expertise is required in designing and using rodent models of neurodevelopmental disorders and in their interpretation.

Animal models offer several advantages in the study of human diseases, allowing researchers to efficiently test hypotheses, control genetic and environmental influences, and perform invasive studies in a manner that is not feasible or ethical in humans. Accordingly, animal models are frequently used in scientific research, including toxicological research, to investigate the causes, mechanisms, progression, and potential treatments of disease. Animal studies have played a key role in identifying the harmful neurodevelopmental effects of drugs already on the market, such as Valproic Acid. Indeed, the FDA specifically looks to animal data to demonstrate the effectiveness of a drug when adequate and well-controlled efficacy studies in humans cannot be ethically conducted (FDA CDER, 2015 "Animal Rule").

Many species can be used to model human disease, and the selection of test species generally depends on several factors (e.g., specific research question, neurobiological system of interest, desired endpoint). Humans and other primates share the vast majority of their genetic information, but primate models are relatively uncommon because they are costly, not widely available, and ethically dubious. Rodents and other small mammals are less resource-intensive and more susceptible to genetic modifications commonly used to model human disease. Rats and mice are frequently used in animal experimentation because they are easy to maintain in the laboratory and can be bred rapidly and at relatively low cost. From a practical standpoint, mice are less expensive and take up less space than larger mammals, allowing researchers to design studies with larger samples. Mice are also easier to genetically manipulate, allowing researchers to perform studies on mice with or without specific genetic characteristics. Rats, on the other hand, typically display more complex social behaviors and therefore can help researchers understand more complex effects of specific compounds. Both rats and mice share a high degree of genetic and physiological concordance with humans. In fact, humans and laboratory rodents are genetically very similar: the genome of humans and mice are approximately 85% identical (Why Mouse Matters, National Human Genome Research Institute, available at https://www.genome.gov/10001345/importance-of-mouse-genome).

Animal models play a crucial role in advancing our understanding of neurodevelopmental disorders, including ASD and ADHD, which are caused by the complex interaction of genetic, epigenetic, and environmental factors. By inducing genetic mutations or exposing animals to toxicants believed to impact neurodevelopment, scientists can observe changes to a myriad of components of the central nervous system, including its neuroanatomical structures, synaptic connectivity, and neurochemical signaling. Animal models can also provide critical information about potential therapeutic interventions and help researchers identify targets for pharmacological interventions. While animal models do not perfectly replicate the complexity of human conditions, they offer a valuable experimental platform for studying neurodevelopmental disorders and aid in the development of new treatments and interventions.

A. ANIMAL MODELS OF ASD

Animal models have been widely used to study ASD. Animal research related to ASD has included research into both the etiology of autism phenotypes (i.e., cause of ASD symptoms) as well as potential targets for pharmacological treatments. Researchers use a variety of animal models, including flies, fish, mice, rats, voles, and non-human primates to mimic the genetic, environmental, and behavioral aspects of ASD in humans. These models allow researchers to ethically study the underlying genetic factors, molecular pathways, and environmental impacts that contribute to ASD. Despite the diversity of species used, mouse models predominate.

The core symptoms of ASD modeled in animals are in social and self-directed behavioral domains. Social behaviors are generally evaluated in social motivation and social communication paradigms; self-directed behaviors are generally evaluated by evaluating repetitive or restricted motor behaviors. Individuals diagnosed with ASD often engage in repetitive stereotyped behaviors. Many mouse models of ASD also engage in repetitive stereotyped behaviors. This can be evaluated by evaluating motor movements such as circling behaviors, the patterns and durations of body grooming behaviors, and the manners in which mice interact with the cages or with novel

objects (Pearson et al., 2010). Several of the most common tests, which are used in several of the studies discussed in this report, are described below.

1. Animal Behavioral Testing and ASD

a. Social Behavior

"Mice have a natural tendency to approach and investigate unfamiliar conspecifics, much like the way a person would greet a stranger. A person with autism often avoids interacting with a stranger, either withdrawing when approached or remaining aloof. Therefore, low social approach in mice represents an endophenotype with a reasonable analogy to the types of social deficits that characterize autism" (Yang et al., 2011).

Three-Chamber Social Test. The three-chamber social interaction test, developed by Dr. Jackie Crawley, a pioneer in behavioral neuroscience, is commonly used to measure social interest and social novelty seeking behavior (Yang et al., 2011). In this test, the subject rodent explores a partitioned arena with a neutral central chamber and two side chambers containing stimuli, as depicted in Figure 19. The sociability phase measures the time spent in the chamber containing a caged rodent versus the chamber containing an empty cage. The social novelty phase measures the time spent in the chamber containing a novel rodent versus the chamber containing the familiar rodent (Bey & Jiang, 2014). Failing to spend more time in the chamber with the mouse vs the empty cage side indicates low interest in peers (associated with ASD's DSM-IV factor I). Likewise, a failure to spend more time in the chamber containing the unfamiliar (novel) rodent compared to the familiar rodent during the social novelty phase indicates a lack of social recognition or social memory (also linked to ASD factor I).

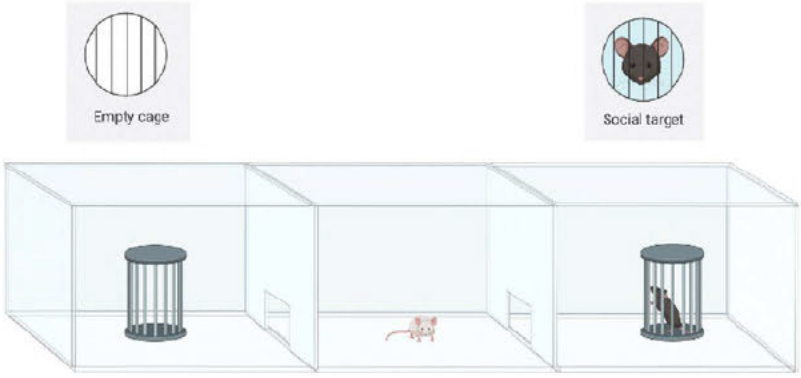

Figure 19. Three-chamber sociability arena.
Created with Biorender.

Direct Interaction Tests. In this test the subject rodent freely interacts with a stimulus rodent and the number, duration, and types of social interactions are measured. Variations of this test include neutral pairing, i.e., the stimulus rodent is an ovariectomized female or juvenile male; or agonistic pairing, i.e., the stimulus rodent is an adult male in the novel arena social (social dyadic) or test rodent's home cage (resident intruder). (Bey & Jiang, 2014). A low number of

interactions and/or little time spent interacting indicate low interest in peers and/or difficulty in maintaining interaction (ASD factor I).

Habituation-Dishabituation Tests. The subject rodent is repeatedly introduced to the stimulus rodent. Decreasing time spent in social interaction represents social recognition of the stimulus rodent. Then, a novel rodent can be introduced as a stimulus rodent, and increased time spent in social interaction represents social recognition. (Bey & Jiang, 2014). Impaired social memory and recognition may indicate low interest in peers and other abnormal social interactions (ASD factor I).

Juvenile Play. Pairs of juvenile rodents are allowed to freely interact, and the number, duration, and types of play behaviors can be measured. (Bey & Jiang, 2014). Low levels of juvenile play and limited duration indicate low interest in peers and/or difficulty maintaining interaction (ASD factor I).

Partition Test. The test rodent explores one-half of a partitioned arena with a novel rodent on the other side of the barrier. The time spent near the partition is a measure of social approach. (Bey & Jiang, 2014). Little or no time spent near the partition indicates low interest in peers (ASD factor I).

b. Communication Behavior

Rodents engage in complex social communication. Rats and mice communicate via vocal, visual, and chemical signals. Such communication between rodents is operationalizable variables relevant to social communicative alterations in neurodevelopmental disorders. The content and quality of the communication can be difficult to determine but the quantity and form of alterations can be quantified through various techniques, for instance:

Adult Ultrasonic Vocalizations. The test male rodent is introduced to a reproductive setting or given access to a stimulus, i.e., estrus female or female urine, or an agonistic setting, i.e., another male rodent (such as in resident-intruder paradigm). The number, duration, and types of ultrasonic calls are recorded (Bey & Jiang, 2014). Fewer and shorter calls may indicate communication deficits (ASD factor II) and may also represent decreased sociability (ASD factor I). Ultrasonic vocalizations can also be measured in females.

Pup Ultrasonic Vocalizations. Young rodents vocalize when away from their mothers. The quality and quantity of these vocalizations can be an important variable. The test rodent pup is briefly separated from its dam and littermates. The number, duration, and types of ultrasonic isolation calls are recorded (Bey & Jiang, 2014). Fewer and shorter calls may indicate communication deficits (ASD factor II) and may also represent decreased sociability (ASD factor I). Increased isolation induced calls can also indicate enhanced alarm signaling and perturbed distress perception in pups that could have relevance to neurodevelopmental impairments perhaps other than ASD.

Scent-Marking. Male rodents place territorial scent marks from multiple anatomic scant glands including facial glands and preputial glands. The test male rodent is exposed to urine from estrus females. The amount and proximity of male urine scent-marks to female urine stimuli is a measure of scent-marking communication behavior (Bey & Jiang, 2014). The number of scent

marks close to stimuli of opposite or same sex may measure olfactory communication (ASD factor II) and/or social interest (ASD factor I) (citations for this from my PhD lab and others e.g., Arakawa et al., 2007; Arakawa et al., 2008; Wohr et al., 2011).

c. *Repetitive/Restricted Behavior*

A hallmark of ASD is restricted repetitive motor behaviors (also colloquially called "stimming" in humans) and circumscribed interests. Self-directed behaviors and restricted and repetitive motor behaviors are measured rather easily in rodents through the tests discussed below and used in several of the studies reviewed here.

Holeboard Exploration. The test rodent explores an arena with a grid pattern of circular holes in the floor allowing for head-dips into the holes. The number and pattern of holes explored is a measure of perseverative exploration (Bey & Jiang, 2014). Perseverative exploration of only one of the holes represents restricted interests and repetitive behavior (ASD factor III).

Marble Burying. The test rodent is introduced to a cage containing marbles atop bedding. The number of marbles buried is a measure of compulsive digging and bedding sifting behavior (Bey & Jiang, 2014). The number of marbles buried are used to represent repetitive, compulsive-like behavior in rodents (ASD factor III).

Maze Reversal Learning. The test rodent must learn a new location of a hidden platform or escape hole in spatial mazes (see below). Its ability to learn a new location is a measure of cognitive flexibility (Bey & Jiang, 2014). An inability to learn the new location represents repetitive behavior and an insistence on sameness (ASD factor III).

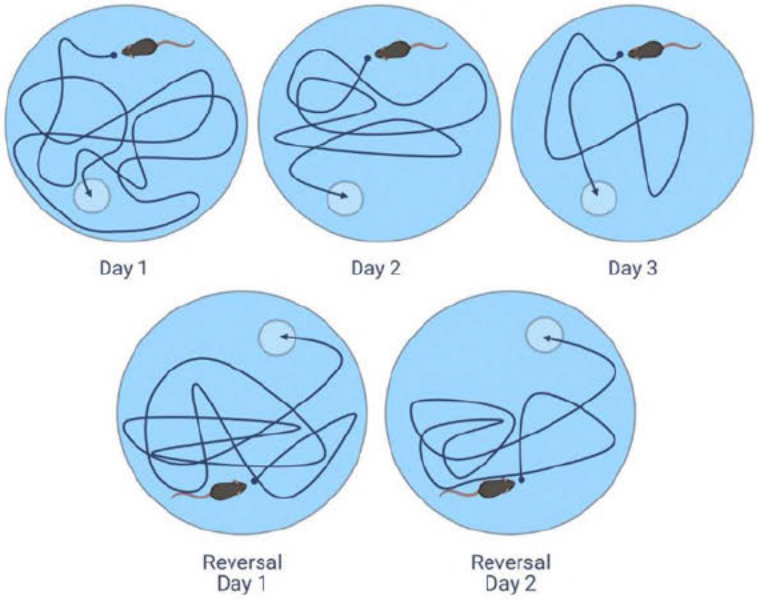

Figure 20. Morris Water Maze & Reversal Learning. Created with Biorender.

Nest Building. The test rodent is given pressed cotton material. The amount of material the rodent shredded and arranged into nest is a measure of nest construction ability (Bey & Jiang, 2014). Shredding (measured relative to weight of intact material) may be representative of repetitive, compulsive-like behavior (ASD factor III).

Repetitive Grooming. The test rodent is observed for self-grooming in its home cage or novel environments. The duration and bouts of grooming are measures of repetitive behavior, while skin lesions are measures of self-injurious behavior (Bey & Jiang, 2014). Grooming and lesions are used to measure repetitive behavior (ASD factor III). The pattern of grooming is highly stereotypical in rodents. In mouse models of ASD, that pattern of grooming can be even more rigidly stereotyped (Pearson et al., 2010).

B. ANIMAL MODELS OF ADHD

Rodent models of ADHD generally attempt to recapitulate attention problems and activity problems that people experience using conditioned and unconditioned behavioral tasks. Spontaneously hypertensive rats exhibit ADHD-like features including inattention and hyperactivity (Sagvolen et al., 2005). Genetic manipulations or dopaminergic brain lesions can also create features of ADHD in rodents.

Animal models of ADHD are somewhat less developed than animal models of ASD, and they have been criticized as not identical to ADHD in humans. However, they remain an essential tool in understanding ADHD behaviors and symptoms in humans, including factors that may ameliorate or worsen those symptoms (Russell, 2005). ADHD models in animals, like ASD models in animals, are not meant to be exact duplicates of these quintessentially human disorders. Instead, scientists seek to better understand symptoms of ADHD which can be readily modeled in animals, including inattention, impulsivity, and hyperactivity.

Genetic manipulation and selective breeding have resulted in several rodent species that are genetic models of ADHD-like phenotypes, including the spontaneously hypertensive rat (SHR) and dopamine transporter knock-out (Dat KO) mouse. SHR models demonstrate face validity in that they are hyperactive, impulsive, and inattentive; they demonstrate construct validity in that they exhibit neuropathological changes similar to those observed in humans with ADHD; and they demonstrate predictive validity in that common ADHD treatments (i.e., psychostimulants and guanfacine) attenuate their hyperactive, impulsive, and inattentive symptoms (Regan et al., 2022). Dat KO (dopamine transporter knockout) mouse models demonstrate face validity in that they are hyperactive, impulsive, and inattentive; they demonstrate construct validity in that Dat KO mice exhibit reduced dopamine reuptake and ADHD in humans is associated with dopamine signaling alterations; and they demonstrate predictive validity in that ADHD treatments, attenuate their hyperactive symptomology (Regan et al., 2022). Variants of this species, including Dat KO heterozygous (Dat KO hets) and DAT Knockdown (Dat KD) are also used to model ADHD (Regan et al., 2022).

Other genetic models of ADHD include the Snap-25 KO coloboma mouse, dopamine receptor KO mice, guanylyl cyclase-C KO mice, and latrophilin-3 KO mice and rats (Regan et al., 2022). ADHD symptoms can be modeled in these rodent species through the tests below, many of which are also used in several of the studies reviewed in this report.

1. *Animal Behavioral Testing and ADHD*

a. *Hyperactivity*

Hyperactivity in animal models is reflected by increased or changed activity during some or all of the activity test or habituation test in an arena or novel cage. Common tests indicating hyperactivity include:

Light/Dark Exploration Box. The test rodent is confined to a dark zone in a box. The rodent is then transferred to a light zone and allowed to commute freely between the two zones, as depicted in Figure 21. Its behavior during the initial confinement and after the transfer is recorded and scored. Unusually high motor activity is treated as indicating hyperactivity associated with ADHD. The light/dark exploration task is generally a test used for evaluating exploration and risk assessment, fear, and anxiety-related behavior of a rodent but it can be utilized for this application as well.

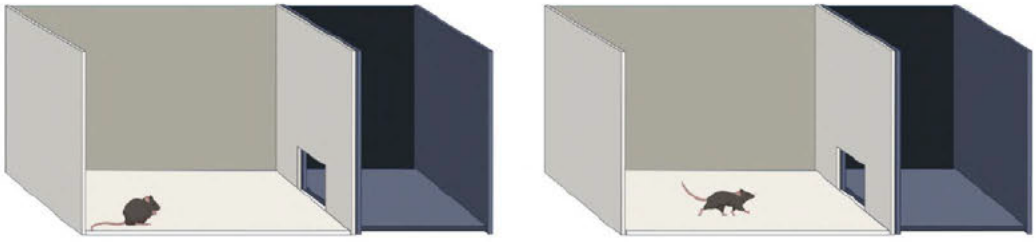

Figure 21. Light/Dark Exploration Box.
Created with Biorender.

Open Field Test. The test rodent is placed in the center or near the walls of the test apparatus for the duration of the test (usually 5 minutes, but up to 60 minutes or more) and target behaviors are measured, including but not limited to horizontal locomotion, rearing or leaning ("vertical activity"), grooming, distance traveled, time spent moving vs. still, and time spent in central zone(s) vs. peripheral zones. Increased activity in the open field apparatus may indicate hyperactivity which is linked with ADHD in humans. A variant of this test is with a stimulant given just before the test to evaluate how sensitive a model is to stimulant-induced hyperlocomotion.

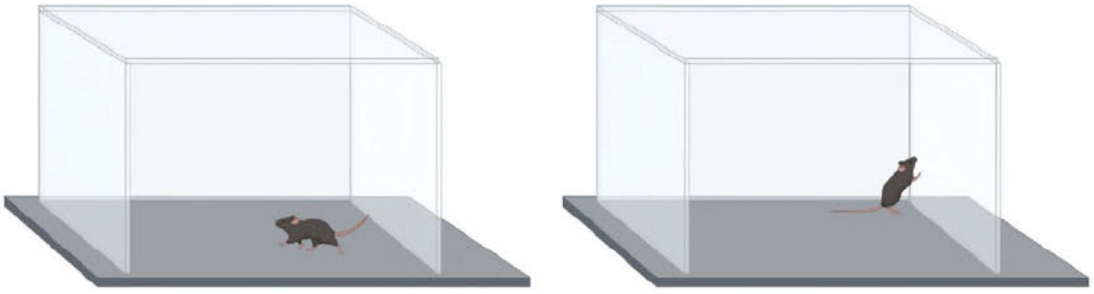

Figure 22. Open Field Test.
Created with Biorender.

Voluntary Wheel-Running Activity. The test rodent is placed in a cage with an attached running wheel. Wheel rotations, running speed, and duration of running are measured as indicators of hyperactivity. This measure can also reveal disruptions to diurnal cycles.

Y-maze. The test rodent is placed in a Y-shaped maze that has three equally-sized radial arms with raised walls and is allowed to freely explore the maze when the test begins. The total number of arm entries and consecutive entries into each arm without revisiting previously-entered arm(s) (known as "spontaneous alternation") are measured. An increased number of arm entries indicates hyperactivity (ADHD factor II) and reduced spontaneous alternation indicates spatial memory deficits, which have been recognized in animal models of ASD and ADHD though it is not a core criterion for either disorder.

b. Impulsivity

Animal models measuring impulsivity, i.e., premature responding and recorded as bursts of responses with short inter-response times, look at the number of responses with short inter-response times to determine the degree of impulsiveness. Common tests to model impulsivity include:

Cliff-Avoidance Reflex (CAR) Test. The test rodent is placed on an elevated platform supported on a rod (similar to a barstool). The rodent's behavior is recorded. The length of time to first fall is calculated as a measure of impulsivity associated with ADHD.

Stop-Signal Reaction Time (SSRT) Test. The test rodent is placed in a chamber and trained to poke first a left lever and then a right lever to secure a reward. But, in 20% of cases, the test rodent receives a stop-signal tone upon pressing the left lever—failure to stop is punished. SSRT measures inhibition of a response that has already been initiated (i.e., the ability to stop). The inability to stop is attributed to impulsivity, and thus associated with ADHD.

T-Maze Test. The test rodent is placed in a T-maze and trained to choose between a small immediate reward and a larger reward that is delayed 30 seconds. Choosing the smaller, but immediate, reward is tied to impulsivity associated with ADHD.

c. Attention

ADHD is associated with problems with attention. In rodents, attention can be evaluated by training animals to respond to cues with varying intensities that require sustained attention. An example is:

5-Choice Serial Reaction Time Task (5CSRTT). As described in my group's study (Baker et al., 2023), mice are trained to watch a touchscreen where one of 5 squares will light up for a brief duration. If they touch the light, they get a food reward. The mice are food restricted so they are motivated to work for the food reward. By measuring how many trials it takes the mice to learn the task one can measure learning. Then, we reduce the duration that the light stays on all the way from 32 seconds to less than one second. Animals that have problems maintaining attention will miss trials and would show fewer correct trials or show more missed (omitted) trials. The task can be made even more challenging by adding distracters or adding punishment if the mice make

a mistake. Also, the number of premature choices that a mouse makes can be indicative of impulsive characteristics.

C. The Role of Animal Model Data in Identifying Causes of Neurodevelopmental Disorders

Animal models of neurodevelopmental disorders like those described above provide critical information to researchers investigating the relationship between environmental exposures and these uniquely human conditions. The developmental neurotoxicity of chemicals and medications can be studied by exposing rodents to the test substance during critical periods of neurodevelopment and measuring the extent to which resulting the behavioral, structural, and/or chemical alterations resemble those found in animal models of NDDs.

Animal studies also allow for the investigation of dose-response relationships between exposures and NDD-like outcomes. By exposing animals to different doses of a chemical or medication, researchers can determine whether there is a dose-dependent relationship between exposure levels and the severity of behavioral, structural, or chemical alterations. This information is helpful for assessing the causative relationship between an exposure and neurodevelopmental outcomes and is key to establishing exposure guidelines. Absence of dose response information (i.e., single dose studies) can nevertheless provide causal and informative information for causal preclinical evidence.

Investigating how developmental exposure to chemicals and medications impact brain structure, neurotransmitter systems, and gene expression in animal testing provides valuable insights into potential pathways and mechanisms through which environmental exposures cause NDDs. The FDA's review produced in this case cites the importance of animal studies to addressing the safety of APAP, noting that it would be "unlikely that further observational studies will provide more clarity without more mechanistic data." While animal studies are not a perfect predictor of pharmaceutical safety in humans, they offer an important tool to examine real-world effects of a compound in a living subject. The makers of Tylenol actively use animal models in their preclinical drug development testing and rely upon results from animal testing (see, e.g., Goethals, 2023).

Animal studies also allow researchers to exert significant control over experimental conditions (e.g., genetics, environment, and exposure). As a result, important variables such as the chemical or medication exposure can be isolated and studied without confounding influences. For example, confounding due to genetic variability can be controlled by experimenting on genetically homogenous subjects (i.e., inbred rodent lines). Laboratory animal experiments can control for the time of day of drug administration and the dosage(s). They normalize other environmental factors such as dietary differences, sexual experience, pathogen burdens, birth order effects, and various other climate, health, housing, psychological and other potential confounding variables.

To illustrate how we can compare species, APAP doses, and evidence of neurotoxicity across humans and mice, I plotted a simple summary of human and mouse oral APAP toxicity. Figure 23 shows oral therapeutic doses for analgesia or antipyresis (in green), doses that could be mildly or moderately hepatotoxic but in a range that the subject can recover (in yellow), and doses that would be lethal without intervention (in orange/red). I then overlayed (in purple) the range of

doses administered to mice in published literature that resulted in neurotoxicological outcomes, whether they be neurotransmission, behavioral changes, relevant transcriptional perturbations or the like (listed below). What is clear is that there are effects at sub-lethal and sub-hepatotoxic doses of APAP.

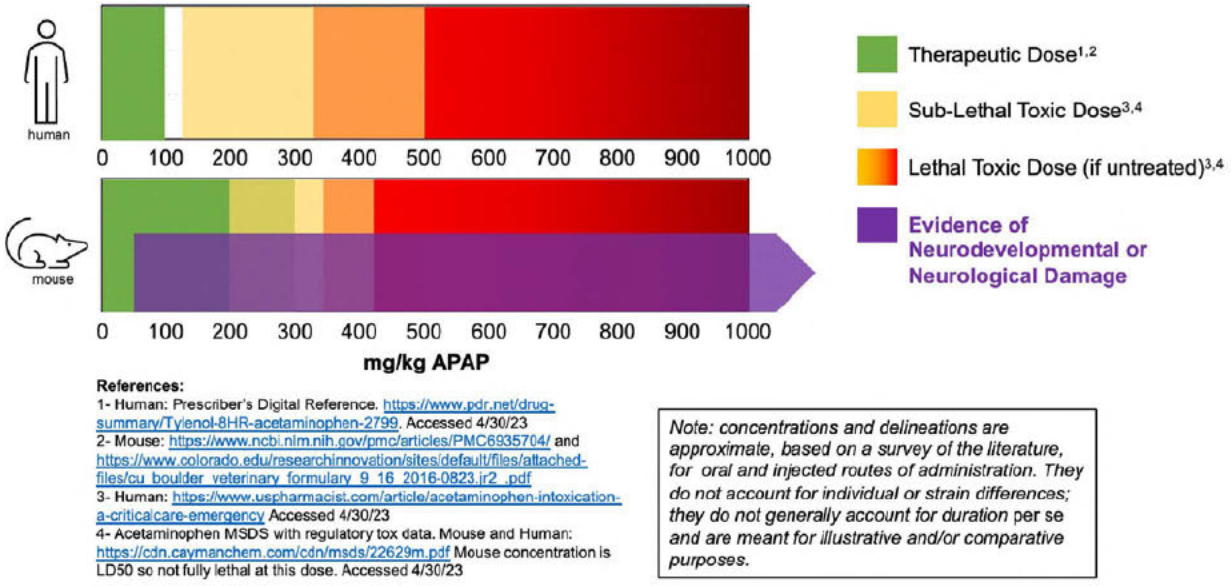

Figure 23. Created for purposes of this report.

At a high level, both mice and rats are essential to preclinical models. Laboratory rodents are ideal for developmental neurotoxicity studies because their reproduction, gestation, and lactation are remarkably similar to humans. Rodents reproduce and develop at a faster rate than larger animals like primates, and because they are smaller, scientists are able to design more, larger and more robust studies. They also engage in intensive parental care, communication, and other social behaviors much like their human counterparts. This is true despite the fact that the gestation and development period of rodents is substantially shorter than that of humans, as depicted in Figure 24, below. For example, gestation in rats is completed within 23 days of conception as compared to 280 days in humans. For mice, the period is approximately 19-19.5 days. With regard to neurodevelopment, post-natal days 3 to 10 in a mouse are equivalent to the third trimester of pregnancy in humans (Philippot et al., 2017). Understanding these differences, scientists can account for them in their study design. Additionally, while animal studies are not a 1:1 comparison to humans, they still provide important, relevant information that can be extrapolated to the human experience.

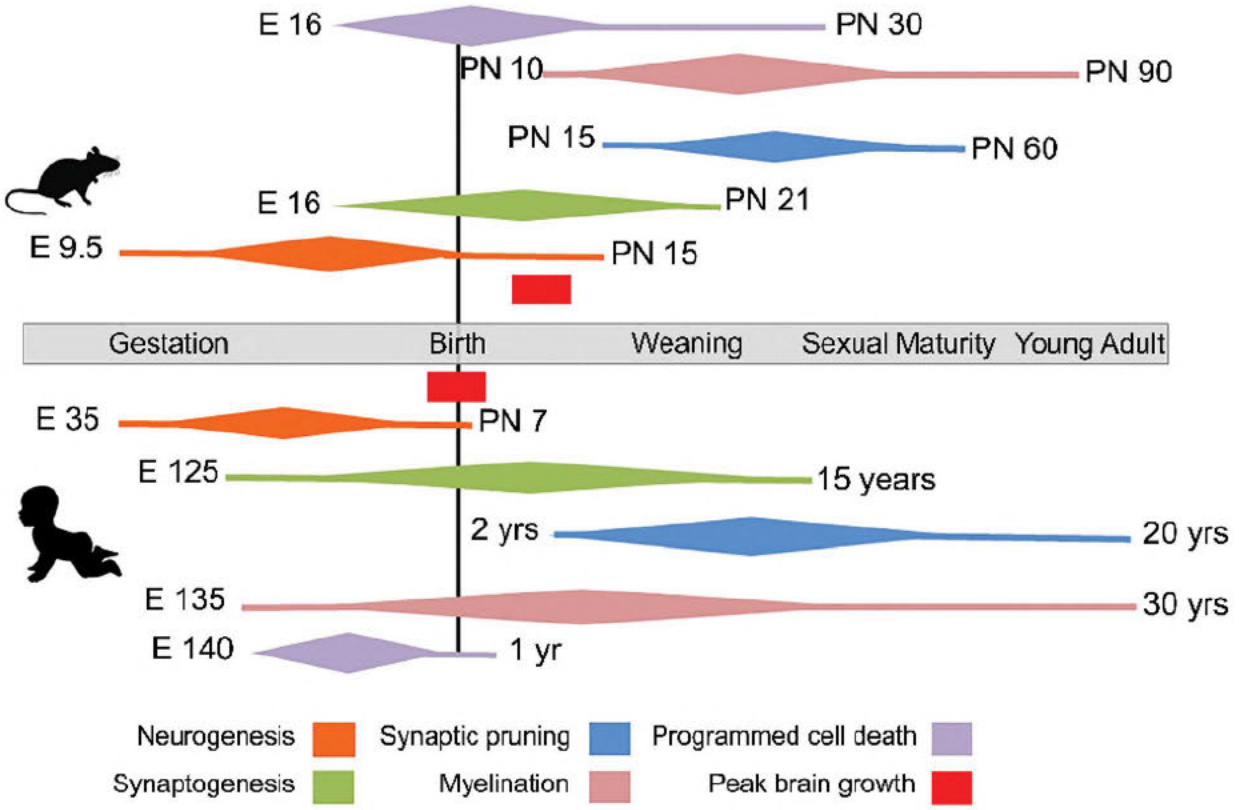

Figure 24. Comparison of brain development milestones of rats relative to humans. Approximate timelines of these processes are shown in relation to anchor events of birth, weaning, sexual maturity, and adulthood. Brain growth spurts are shown in red. Individual processes are color-coded, with peak activity indicated by the widest portion of the diamond. Rats and mice are broadly equivalent in these milestones.
Reproduced from Zeiss, 2021.

Peak brain growth, neurogenesis, and programmed cell death that is complete or nearing completion at term in a human neonate persists into the first 2-3 postnatal weeks in a rodent (Pressler & Auvin, 2013). Accordingly, preclinical APAP neurotoxicology researchers may choose to include this 3rd trimester human brain period equivalent in their translational exposure design.

For example, in Philippot et al., 2017, groups of mice were administered either saline or two subcutaneous injections of 30 mg/kg APAP four hours apart on post-natal day (PND) 3, PND 10, or PND 19. Mice underwent behavioral testing at 2 months of age which corresponds to early adulthood in humans. The mice that were administered APAP on PND 3 and 10 showed significant changes in spontaneous behaviors compared to control mice, whereas those administered on PND 19 did not. Though administered to mice postnatally, the results are clinically relevant to a human fetus exposed to a therapeutic dose of APAP during the third trimester. The results of this study, which are discussed in more detail later in this report, show that APAP can perturb neurodevelopment during multiple critical stages, and is most damaging prenatally as opposed to postnatally.

In addition, animal study designs allow researchers to explore a molecule's effect on genes and gene expression. As discussed in more detail below, current research techniques allow scientists to examine epigenetic changes in response to toxic substances. Changes to the epigenome can alter gene expression and can help us understand complex neurodevelopmental outcomes. One way to monitor epigenetic changes is to examine DNA methylation. DNA methylation is a chemical modification that can silence or regulate the expression of genes. DNA methylation is affected by factors including exposures to environmental toxins and medications. DNA methylation analysis can allow us to identify specific genes affected by a chemical. Histone modification offers another method by which we can examine the effect of environmental exposures on gene expression. Histone proteins are used to package DNA into chromosomes. Modification to histones, like modifications in DNA methylations, can alter gene expression. Histology and microscopy techniques, including the use of immunomarkers,[6] in immunohistochemical techniques also allow us to examine evidence of changes in protein levels, structural changes in tissue, DNA and other molecular damage, cell composition changes, and even cell death. Histological and staining approaches can be used to evaluate the size of structures and cells as well as their abundance and arrangement. These techniques are like what is often done by pathologists evaluating human tissue to diagnose disease or determine causes of death and disability.

Animal study designs can also use comprehensive molecular "omics" tools such as genomics, transcriptomics, metabolomics, methylomics, or proteomics. [Adding "omics" to the end of a term simply means that the researcher is capturing the entirety of something]. Omics techniques are one of the most powerful tools at our disposal to understand the effect of toxicants on complex health outcomes. Omics refers to a collection of high-throughput techniques that allow researchers to perform comprehensive analysis of molecules and molecular processes, including genes, proteins, and metabolites. Omics analyses allow researchers to better understand biological processes and ways they can be disturbed on both a larger and more detailed scale (Figure 25, below). In my research group's study, addressed in more detail later in this report, we used transcriptomics techniques to show the effect of APAP on a multitude of specific genes.

[6] An immunomarker, also referred to as an immunohistochemical marker or immunohistochemistry (IHC) marker, is a substance or molecule employed to identify and visualize particular proteins or antigens within cells or tissues. These immunomarkers find extensive application in research, diagnostics, and pathology for the identification and characterization of cellular components, exploration of disease mechanisms, and assistance in diagnosing and classifying various conditions.

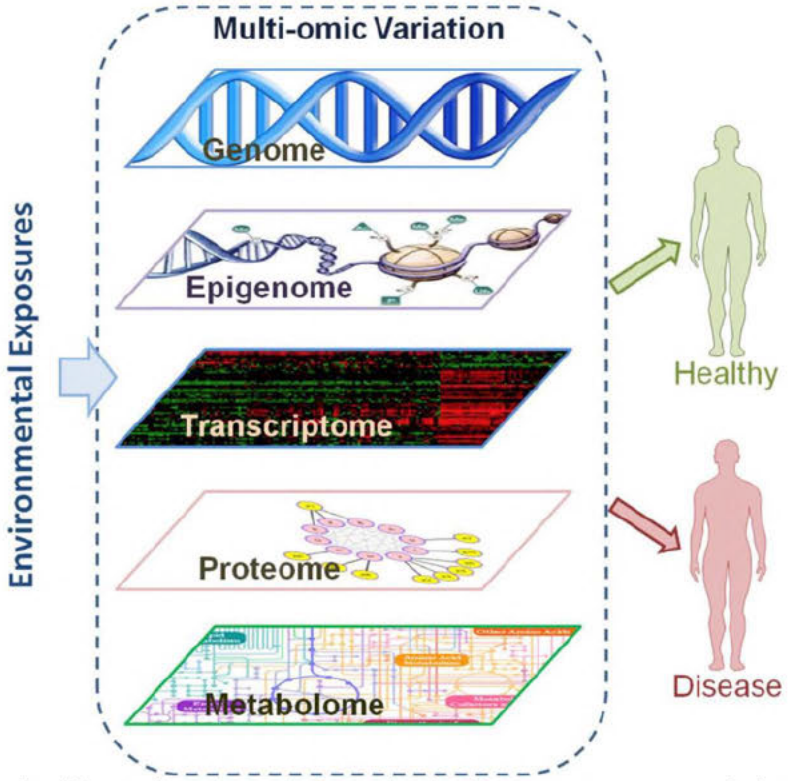

Figure 25. Levels of "omics" analyses. At the top level is the genome, etc, to metabolome which is the comprehensive evaluation of all metabolites in a biospecimen. Modern systems biology tools integrate across these levels of analysis to understand exposures (left) to health and disease states (right). This concept is critical to understanding causal roles of prenatal APAP exposure and neurodevelopmental disorder outcomes. The weight of evidence should rely substantially on untargeted omics techniques.
Reproduced from Sun & Hu, 2016.

Analytical techniques are often necessary to measure biomolecules. Proteins and hormones can be quantified using Western Blot or Enzyme-linked immunosorbent assay (ELISA). HPLC-EC assay refers to a specific analytical method that combines High-Performance Liquid Chromatography (HPLC) with Electrochemical (EC) detection. It is commonly used to quantify and analyze compounds that can undergo electrochemical reactions, such as neurotransmitters, catecholamines, or other electroactive substances. Published studies list the specific analytical tools used and how they are validated. Best practices are agreed upon in the disciplines of study and most academic journals have standards of reporting for the validation and quality standards for such techniques.

X. APAP'S MECHANISMS OF NEURODEVELOPMENTAL INJURY

Understanding the mechanisms by which prenatal APAP exposure leads to neurodevelopmental disorders in offspring helps understand the causal relationship between prenatal APAP use and the symptoms of neurodevelopmental disorders, including ASD and ADHD. The studies analyzed and discussed in this report reveal multiple complex and intermingled mechanisms of action. My discussion below focuses on the mechanisms of action that are most central to the impact of APAP on human neurodevelopment from my perspective as a neurotoxicologist.

A. APAP Causes Formation of a Toxic Metabolite, Inducing Oxidative Stress and Mitochondrial Inhibition

In my opinion, based on my experience studying APAP and my weighing of the preclinical data as discussed below, the primary mechanism by which APAP causes neurodevelopmental disorders is through oxidative stress caused by the creation of a reactive metabolite NAPQI and the depletion of glutathione (GSH). Oxidative stress refers to a state of imbalance between the production of reactive oxygen species (ROS) and the capacity of an organism to detoxify and repair the damage induced by these species. ROS are highly reactive molecules that can cause damage to cellular components including proteins, lipids, and DNA. While ROS are naturally produced as byproducts of normal cellular metabolism, excessive amounts can lead to cellular damage and dysfunction. That dysfunction may manifest as DNA damage, cell death, and dysfunction. During brain development, these processes can cause disruptions in neurodevelopment leading to outcomes including symptoms of ASD and ADHD.

1. Oxidative Stress

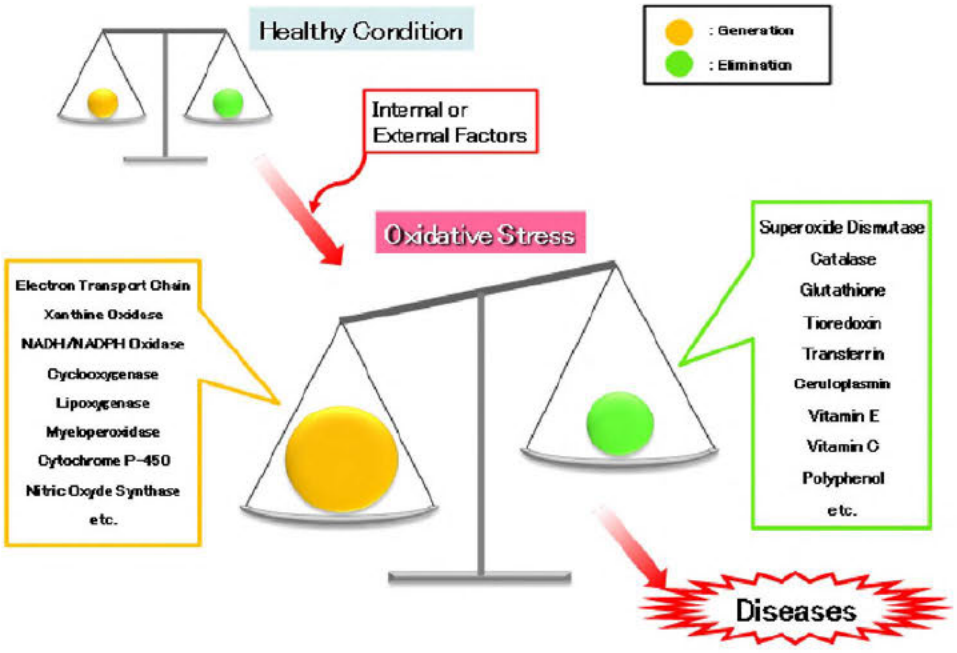

Figure 26. Graphic depicting oxidative stress as an imbalance of free radicals and antioxidant and detoxification systems. Oxidative stress is depicted as a left leaning imbalance via free radical sources outweighs antioxidant capacity. Reproduced from https://www.graphyonline.com/archives/IJPNC/2015/IJPNC-103/index.php?page=figure&num=1.

Oxidative stress refers to the imbalance of pro-oxidants (free radicals such as reactive oxygen and reactive nitrogen species) and antioxidants. The figure above provides an overview of how cells and systems maintain a balance of free radicals that are formed from metabolic systems such as the mitochondrial electron transport chain that are balanced by the presence of antioxidant and detoxification systems. It is well established that APAP causes oxidative stress. As discussed previously in this report, APAP is rapidly metabolized upon ingestion through a series of enzymatic reactions. One of the primary metabolic pathways involves the formation of a toxic

metabolite, NAPQI, through the action of the cytochrome P450 enzymes (specifically the enzyme cytochrome P450 2E1, also called CYP2E1). Importantly, CYP2E1 is expressed in the prenatal brain (Brzezinkski et al., 1999) including as early as 2nd trimester with higher levels of expression in the 3rd (O'Hara et al., 2015).

Under normal circumstances, NAPQI is rapidly detoxified by reduced glutathione (GSH). GSH is an important antioxidant molecule present in cells throughout the body that plays a crucial role in mitigating oxidative stress and maintaining cellular redox balance. During periods of increased oxidative stress, the demand for GSH increases as it is utilized to neutralize ROS and counteract the damaging effects. However, prolonged or excessive oxidative stress can deplete GSH levels, leading to a compromised antioxidant defense system. If the level of APAP exceeds the liver's GSH reserves, NAPQI can accumulate, leading to oxidative stress. Without sufficient GSH reserves, the body is left without a defense to the toxic effects of NAPQI.

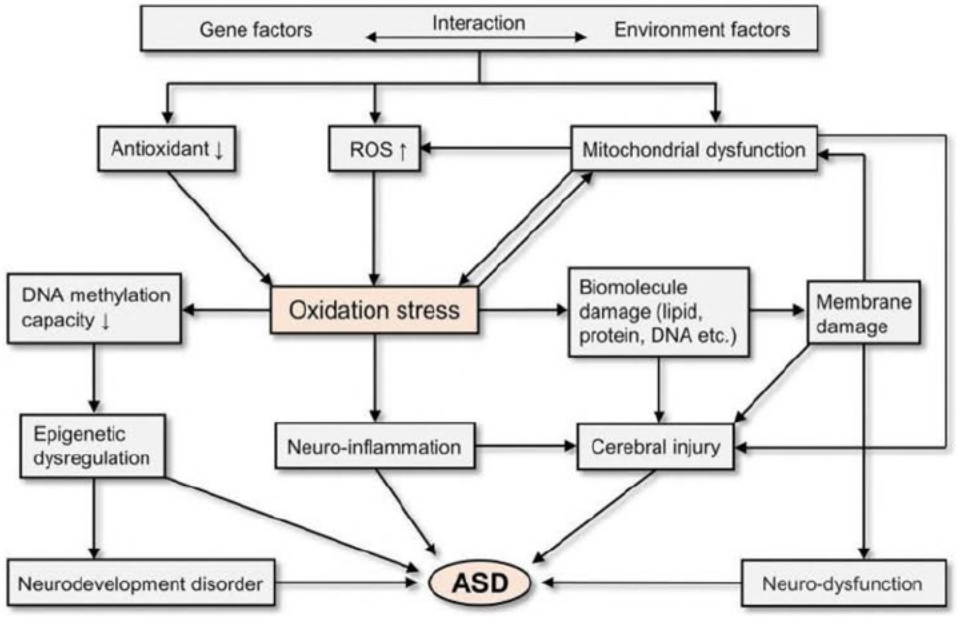

Figure 27. Graphical depiction of the ways oxidative stress can perturb neurodevelopment and lead to neurodevelopmental disorders.
Reproduced from Liu et al., 2022.

A number of techniques can be used to quantify and measure levels of oxidative stress caused by individual compounds, including in silico, in vitro, and in vivo studies. Even in human subjects, biomarkers can be used to demonstrate the level of oxidative stress caused by compounds including APAP. 8-hydroxy-2'-deoxyguanosine (8-OHdG) is a specific biomarker of oxidative stress used in both human and animal studies. In humans, higher concentrations of APAP have been shown to be associated with higher 8-OHdG in the umbilical cord (Anand et al., 2021). Higher urinary concentrations of APAP are also associated with higher levels of oxidative stress biomarkers including 8-OHdG (Li et al., 2022).

In the brain, oxidative stress can significantly impact neurodevelopment, particularly during the critical periods of early brain development. In general, the brain is particularly susceptible to oxidative stress due to its high metabolic rate and oxygen consumption. During

neurodevelopment, oxidative stress can result in damage to developing neurons and their supporting cells, leading to impaired brain function and cognitive deficits. Additionally, excessive ROS production can trigger inflammation and cause further damage to developing brain cells. Numerous studies have demonstrated a link between oxidative stress and neurodevelopmental disorders, including ASD and ADHD (Curpan et al., 2021; Hayashi et al., 2012; Liu et al., 2022; Nishimura et al., 2021; Usui et al., 2023; Wells et al., 2009). In individuals with these disorders, there is evidence of increased oxidative stress and decreased antioxidant defense systems in the brain, which may contribute to their development and progression. Other neurotoxic substances known to cause neurodevelopmental outcomes, including lead, also show evidence of causing oxidative stress on the developing brain (Nishimura et al., 2021).

Oxidative stress caused by APAP can also leave a developing brain vulnerable to other causes of oxidative stress including other toxic exposures. The reverse is also true. Individuals with low detoxification abilities because of, for example, lower GSH levels, could show amplified toxicity in response to APAP, show longer persistence of NAPQI radicals and acquire more damage from oxidative stress. This is particularly relevant here, where research suggests that humans have less GSH when pregnant than when non-pregnant (Balasubramanian & Birundha, 2019). Accordingly, pregnant people may have less ability to detoxify NAPQI which could cause maternal cellular damage which could cause inflammatory responses that signal damage to the fetal brain development.

As discussed previously in this report, CYP2E1 is expressed at various times in various locations in the brain. The varying locations of CYP2E1 expression in the fetal brain over the course of development explains the heterogeneity of outcomes in APAP-exposed animals and humans. Because the brain changes every day during in utero development, small changes in terms of the timing of exposure could lead to different parts of the brain being exposed to NAPQI as CYP2E1 expression changes dynamically over time.

2. Mitochondrial Damage

APAP's toxic metabolite, NAPQI, can also damage mitochondria, the organelles which produce adenosine triphosphate (ATP), the main source of energy in cells. Normal mitochondrial function is critical for normal brain development, in part because the brain is a very energy-intensive organ and requires a great deal of ATP to function normally.

Oxidative stress can damage mitochondria. Mitochondria also have their own DNA, separate from the nuclear DNA present in the cell, and oxidative stress can damage mitochondrial DNA, leading to mutations and impaired function. Mitochondrial DNA, also called mtDNA, is particularly vulnerable to oxidative damage because it is located near the site of ROS production during oxidative phosphorylation. Oxidative stress can cause mtDNA mutations, deletions, and strand breaks, leading to impaired mitochondrial function and energy production. Oxidative stress can also dysregulate proteins involved in mitochondrial processes, affecting their structure and function. In addition, oxidative stress can lead to lipid peroxidation, which can disrupt mitochondrial membranes, and compromise their integrity. Dysregulation of calcium levels within mitochondria can occur, further affecting mitochondrial function.

Once bound to the proteins in mitochondria, NAPQI can result in decreased ATP, leading to a cascade of consequences ranging from additional formation of reactive oxygen nitrogen species, altered gene expression, and apoptosis.

3. Oxidative Stress and Inflammation

In addition to its own effects, oxidative stress also has a complex and bi-directional relationship to inflammation. When neuroinflammation (inflammation of the brain) occurs, immune cells are activated, and a release of pro-inflammatory molecules occurs. Oxidative stress can promote neuroinflammation by activating signaling pathways that induce the production of pro-inflammatory molecules, such as cytokines, chemokines, and reactive oxygen species. These inflammatory mediators can further perpetuate oxidative stress. Accordingly, inflammation and oxidative stress do not act alone. Given the early life neuroinflammatory etiology of ASD and ADHD, any stressor that can cause oxidative stress and/or inflammatory signaling has the potential to trigger the cellular and synaptic changes that underlie ADHD.

4. Toxic and Therapeutic Properties of APAP are Contextual

APAP can show neuroprotection and anti-inflammatory influences when used alongside other agents or manipulations. APAP might also exert helpful effects at small doses, such as improvements in learning or reductions in anxiety at low concentrations. However, it is critical that those effects be viewed in context of potentials for disturbances to developmental programs during gestational brain development. In the context of existing damage or stress, APAP can also act therapeutically to remove pain and reduce the propagation of damage in specific contexts. However, APAP is also capable as acting as the trigger of inflammation and oxidative stress. When APAP is administered at lower doses and durations to an animal in a disease state, its effects can activate the body's ability to produce antioxidants. However, that protective effect can eventually give way to the inflammation, oxidative stress and other toxic processes caused by APAP. These considerations are important insofar as arguments to the safety of APAP to the developing brain cannot be derived from studies showing that APAP improves outcomes when there is a major stressor or insult, such as studies where there is a brain lesion (e.g., APAP improves brain recovery when there is an ibotenic acid lesion i.e., Leroux et al., (2010)). In other words, although APAP is therapeutic under certain conditions of damage in the nervous system, it does not mean it does not cause harm in other health or disease states.

B. DNA Damage, Epigenetic Changes and Cell Death

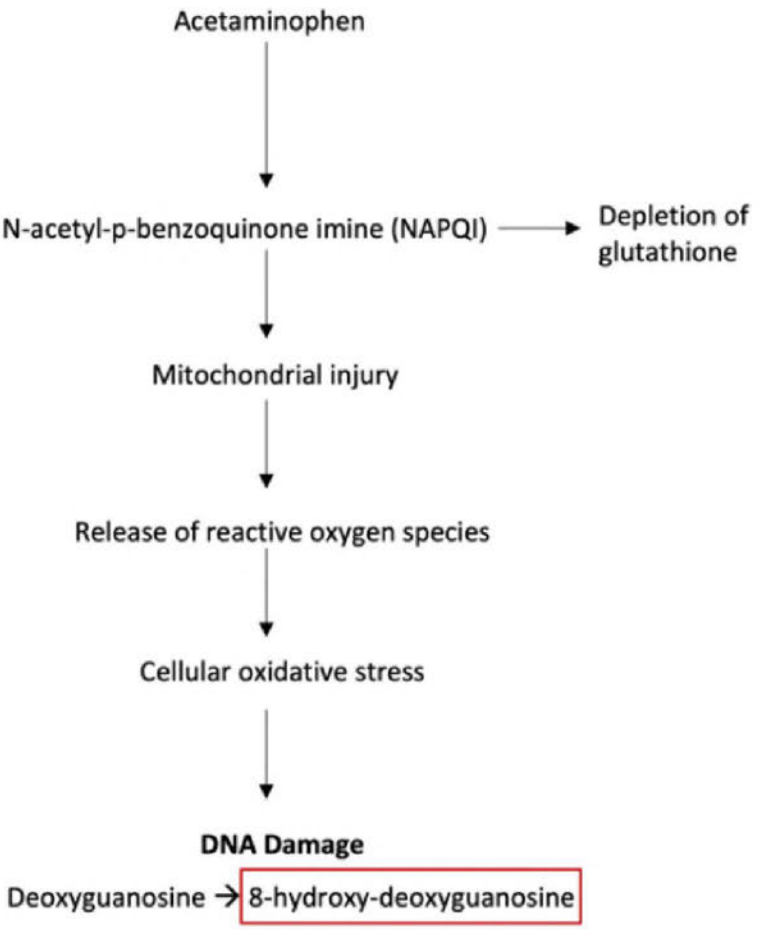

Figure 28. Pathways from APAP exposure to DNA damage. Reproduced from Anand et al., 2021.

1. Cellular Stress Effects on Neuronal Function and Neurodevelopment

Oxidative stress and inflammation lead to reduction in synaptic gene expression and can lead to alterations in cell migration, stem cell and progenitor pool maintenance and differentiation, and changes to neuronal expression of specialized neuronal gene programs. Neurons are particularly avoidant to programmed cell death (Kole et al., 2013), in part, because they are cell types that cannot replace themselves. In my ten years of studying gene transcriptional responses of nervous system cells and tissues to stressors such as drugs and environmental chemicals, my colleagues and I have noted that neurons and brains will downregulate genes that are specialized to neurons, such as synaptic genes, and genes that are long (King et al., 2013; Zylka et al., 2016). Most long genes happen to be synaptic genes. By downregulating long synaptic genes, neurons can protect themselves from damage associated with oxidative stress and instead direct limited energy towards transcriptional programs to detoxifying and antioxidant responses (Pearson et al., 2016). APAP causes pronounced oxidative stress and mitochondrial inhibition as described in the section above. This would lead to the expression signature as shown in the figure below, in cluster